



# Identification and genotyping of *Enterocytozoon bieneusi* among human immunodeficiency virus infected patients

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## KEYWORDS

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Phylogenetic analysis

**Summary** Microsporidia cause diarrhea among human immunodeficiency virus (HIV) infected patients worldwide. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most common species infecting HIV patients. Various genotypes of *E. bieneusi* are transmitted from human to human (anthroponotic route) or from animal to human (zoonotic route). However, there is no study from India on genotypes of *E. bieneusi* among infected hosts. Therefore, we aimed to (a) study the prevalence, clinical symptoms, and species identification of microsporidia among HIV infected patients and (b) perform a genotypic analysis of *E. bieneusi* and a phylogenetic interpretation of the transmission of different genotypes among infected hosts. Two hundred and twenty-two HIV-infected patients and 220 healthy controls (HC) were tested for the presence of microsporidia using modified trichrome (MT) staining and PCR. Demographic, clinical and laboratory parameters were studied. Species identification was performed using PCR-RFLP. All *E. bieneusi* isolates were subjected to genotypic and phylogenetic analysis. Patients with HIV [ $n=222$ , age  $37.4 \pm 10.4$  y, 169 (76%) male] were more commonly infected with microsporidia than the HC [ $n=220$ , age  $34.5 \pm 6.5$  y, 156 (71%) male], using MT stain and PCR [4/222, 1.8% vs. 0/220,  $p=0.04$ ]. Patients infected with microsporidia more commonly presented with diarrhea than those not infected with microsporidia [4, 100% vs. 98/218, 45%;  $p=0.04$ ]. *E. bieneusi* was detected in all patients with microsporidia. Four novel genotypes (Ind1 to Ind4) were identified. Ind1 showed 95%

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similarity with genotype L (AF267142.1) reported in cats (Germany). Genotypes Ind2 to Ind4 showed 94–96% similarity to host-specific genotype A (AF101197.1) reported in humans. Phylogenetic analysis mainly showed an anthroponotic route of transmission (3/4), while the zoonotic route (1/4) was also observed. The prevalence of microsporidia among HIV-infected patients was 1.8%. Patients with microsporidia commonly present with diarrhea. *E. bienersi* is the most common species infecting the study population. Four novel genotypes of *E. bienersi* were identified, suggesting presumptive transmission mainly through the anthropological route.

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## Introduction

Microsporidia commonly cause intestinal microsporidiosis among HIV-infected patients worldwide, with a prevalence ranging from 0.1% to 50% [1]. It is still under-reported in HIV patients from a developing country such as India, with a prevalence ranging from 1% to 15.9% [2–5]. Diarrhea is the most common clinical manifestation of intestinal microsporidiosis among HIV patients with CD4 cell counts  $\leq 100$  cells/ $\mu$ l [6,7].

*Enterocytozoon bienersi* (*E. bienersi*) and *Encephalitozoon intestinalis* are the most common species of microsporidia causing intestinal microsporidiosis in humans [5]. Intestinal microsporidiosis caused by *Encephalitozoon hellem* has been diagnosed in very few cancer patients [8]. *E. bienersi*, the most common species, is often associated with chronic diarrhea in patients infected with HIV [6]. The drug of choice, albendazole, is not very effective against *E. bienersi*. Therefore, emphasizing strategies to prevent infection in humans would be useful. The establishment of preventive measures depends upon tracing the route of transmission. Spores of *E. bienersi* infecting humans, animals and birds are shed in the host's feces. Therefore, the transmission route may be person to person (anthroponotic) or animal to human (zoonotic). Genotyping of *E. bienersi* isolates is a valuable tool for epidemiological investigations [9]. The genotyping is widely based on analysis of an internal transcribed spacer (ITS) of rRNA genes of microsporidia. To date, approximately 90 genotypes of *E. bienersi* infecting humans and animals have been identified [10]. Some of the genotypes are host specific, infecting only humans, while others can infect humans as well as animals and thereby have zoonotic potential [11]. *E. bienersi* is the most common species infecting animals and humans. Its transmission route among humans is still unclear in a developing nation such as India. To date, no study has

been reported on genotypes of *E. bienersi* among HIV-infected patients in India. Understanding the molecular epidemiology of *E. bienersi*, including the route of transmission, would help to establish prevention strategies in India.

Accordingly, we aimed to (a) study the prevalence, clinical symptoms and species identification of microsporidia among HIV-infected patients and (b) perform genotypic analysis of *E. bienersi* and phylogenetic interpretation of the transmission of different genotypes among infected hosts.

## Methods

### Study population and protocol

Two hundred and twenty-two consecutive HIV-infected patients (with and without diarrhea) attending the Immunology unit of a tertiary care institution were tested for the presence of microsporidia during March 2010 to November 2014. Patients infected with HIV were included in the study as per National AIDS Control Organization (NACO) guidelines [12]. Patients without HIV infection were excluded. Two hundred and twenty age- and sex-matched healthy controls without any history of apparent illness within the last 4 weeks were included in the study. Data on demographic, clinical and laboratory parameters were recorded in a standard questionnaire for each patient. Patients were asked if they purify potable water (filtered water) or consume the potable water without any purification (unfiltered water). The study protocol was approved by the Institutional Ethics Committee (PGI/DIR/RC/1085/2007).

### Sample collection

Three consecutive fresh stool samples were collected from each patient and healthy control. The samples were subjected to routine stool microscopy

(normal saline and iodine), and a part was stored at  $-40^{\circ}\text{C}$  in normal saline for deoxyribose nucleic acid (DNA) extraction. Blood samples were also collected in an ethylene diamine tetraacetic acid (EDTA) vial for CD4 count.

### Sample processing

Stool samples were subjected to the formalin ether concentration technique. Thereafter, smears were made from the deposit and air-dried. These smears were stained using Weber's modified trichrome stain with certain modifications [13]. Briefly, fast green was used as a counter-stain, and a smear was incubated in trichrome stain at  $50^{\circ}\text{C}$  for 10–12 min. The smears were observed under a light microscope. CD4<sup>+</sup> cells were counted using Becton Dickinson (BD) fluorescence-activated cell sorting (FACS) flow CD4/CD3 reagent kit (BD Biosciences, CA, USA).

### Species identification

Three consecutive stool samples collected from each patient and healthy control were pooled into one aliquot each. DNA was extracted from the pooled stool samples using a QIAamp Qiagen mini stool kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions with some modifications. Briefly, the sample was suspended in phosphate-buffered saline (PBS) and subjected to centrifugation. The suspension was heated at  $80^{\circ}\text{C}$  for 10 min. The extracted fecal DNA was subjected to amplification of the conserved region of a small subunit of the rRNA (SSU rRNA) gene of microsporidia using a previously published forward primer C1 (5'-CACCAGGTTGATTCTGCC-3') and reverse primer C2 (5'-GTGACGGGCGGTGTGTAC-3') and polymerase chain reaction (PCR) cycle conditions [14].

The amplified fragments were subjected to digestion using the restriction endonucleases HinfI and HindIII to differentiate among four human microsporidia species, namely, *E. bieneusi*, *E. intestinalis*, *E. cuniculi* and *E. hellem* [14].

### Detection criteria

Detection of spores of microsporidia using microscopy in one or more stool samples or its DNA during molecular analysis indicated that the sample was positive for microsporidia.

### Genotyping of *E. bieneusi*

Genotyping of *E. bieneusi* was performed through nucleotide sequence analysis of the ITS region of the rRNA gene. To amplify the ITS region of the rRNA gene, two-step nested PCR was performed. For the primary PCR, a PCR product of 410 bp was amplified using primers AL4037 5' GATGGTCATAGGGATGAAGAGCTT 3' and AL4039 5' ACGGATCCAAGTGATCCTGTATT 3'. The PCR mixture consisted of 1 or 2  $\mu\text{L}$  of DNA, 200  $\mu\text{M}$  (each) deoxynucleoside triphosphates, 1 $\times$  PCR buffer (Perkin-Elmer, Foster City, CA), 3.0 mM  $\text{MgCl}_2$ , 5.0 U of Taq polymerase (GIBCO BRL, Frederick, MD) and 200 nM (each) primers in a total of 100  $\mu\text{L}$  of reaction mixture. The reactions were performed for 35 cycles ( $94^{\circ}\text{C}$  for 45 s,  $55^{\circ}\text{C}$  for 45 s and  $72^{\circ}\text{C}$  for 60 s) with an initial hot start ( $94^{\circ}\text{C}$  for 3 min) and a final extension ( $72^{\circ}\text{C}$  for 10 min). For the secondary PCR, a fragment of 392 bp was amplified using 2.5  $\mu\text{L}$  of primary PCR product and primers AL4038 5' AGGGATGAAGAGCTTCGGCTCTG 3' and AL4040 5' AGTGATCCTGTATTAGGGATATT 3'. The conditions for secondary PCR were identical to those of primary PCR [15].

### Sequencing

Purified PCR products were sequenced by commercially available sequencing services, Bangalore Genei, Bangalore, India using *E. bieneusi* specific ITS sequence forward primer. Electropherograms were analyzed and aligned with previously reported ITS sequences of *E. bieneusi* using standard software (Chromas program, Technelysium Pvt. Ltd., Sydney, Australia and Bio Edit v 7.0.5, Ibis Biosciences, Carlsbad, California, respectively). Identified sequences were submitted to the National Center for Biotechnology Information (NCBI).

### Phylogenetic analysis

*E. bieneusi* ITS sequences were determined, and a multiple alignment was performed using the ClustalW program (<http://www.clustal.org/>). Phylogenetic analysis was performed using the MEGA 5.0 program employing a distance matrix and a maximum-likelihood parameter. A neighbor joining tree was generated using the Kimura two-parameter model.

### Statistical analysis

Data were checked for normal distribution using the Kolmogorov–Smirnov test. Categorical, parametric and non-parametric continuous data were

presented as proportion, mean, standard deviation, median and inter-quartile range, respectively. Chi-square, Fisher's exact, and Mann–Whitney *U* tests were used to compare between categorical, continuous parametric and non-parametric data, respectively. *P* values below 0.05 were considered significant for all statistical analysis. Statistical analysis was performed using SPSS version 15 (SPSS, Inc., Chicago, IL, USA).

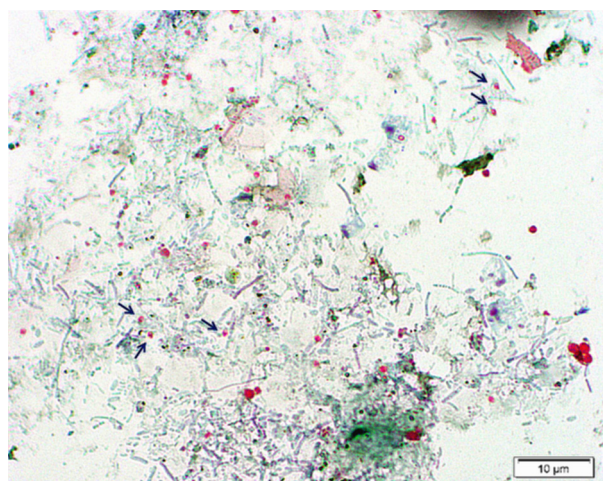
## Results

### Study population

A total of 660 stool samples were collected from 222 HIV-infected patients. Of the 222 HIV-infected patients, 169/222 (76%) were male, with a mean age of  $37.4 \pm 10.4$  y. Of the 220 healthy controls, 156/220 (71%) were male, with a mean age of  $34.5 \pm 6.5$  y.

### Detection of microsporidia among HIV-infected patients and controls

Four of 222 (1.8%) HIV-infected patients and none of the healthy controls had microsporidia ( $p = 0.04$ ) using modified trichrome stain (Fig. 1) and PCR.



**Fig. 1** Spores of microsporidia in a stool sample of an HIV-infected patient under 1000 $\times$  magnification in a light microscope. The arrow represents the spores of microsporidia.

### Factors associated with the occurrence of microsporidia among HIV-infected patients

Table 1 shows the factors associated with the occurrence of microsporidia among HIV-infected patients. All patients infected with microsporidia were male, with a mean age of  $36 \pm 5.4$  y. Diarrhea was more prevalent among patients infected with microsporidia than those without (4/4, 100% vs. 98/218, 45%;  $p = 0.04$ ). Data on the type of

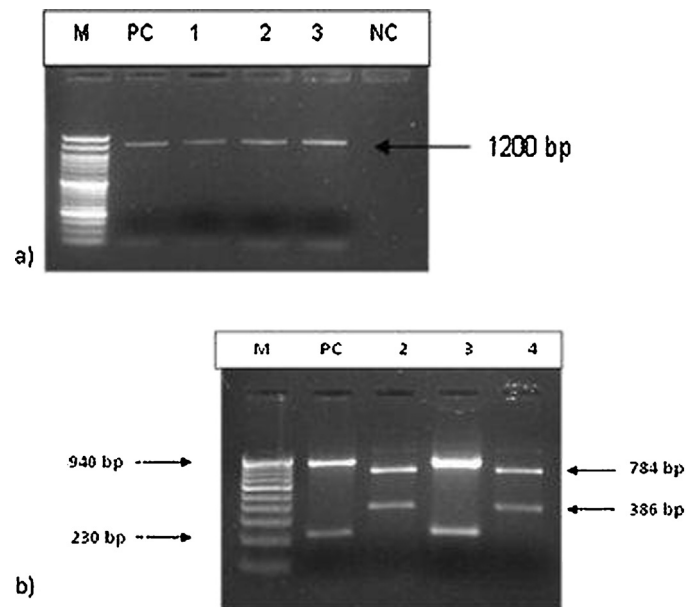
**Table 1** Demographic, clinical and laboratory parameters of patients with and without microsporidiosis among HIV infected patients.

	Microsporidia present ( <i>n</i> = 4)	No microsporidia ( <i>n</i> = 218)	<i>p</i> -Value
Age (mean, SD)	36 (5.4)	37.5 (10.5)	0.86
Gender (male)	4 (100%)	165/218 (75.7%)	0.25
Diarrhea	4 (100%)	98/218 (45%)	0.04
Type of diarrhea <sup>a</sup>			
Acute	2/4 (50%)	32/98 (32.7%)	0.5
Persistent	1/4 (25%)	22/98 (22.4%)	0.9
Chronic	1/4 (25%)	45/98 (46%)	0.4
Daily stool frequency (median, IQR)	4 (2.5, 4)	2 (2, 4)	0.3
Stool character (watery)	2 (50%)	86 (39.4%)	0.6
CD4 count			
<200 cells/ $\mu$ l	4/4	155	0.4
200–500 cells/ $\mu$ l	0	55	
>500 cells/ $\mu$ l	0	8	
Fever	2/4 (50%)	124/218 (56.9%)	1.0
Cough	2/4 (50%)	72/218 (33%)	0.6
Contact with animals <sup>b</sup>	2/4 (50%)	2/93 (2.1%)	0.3
Type of drinking water <sup>b</sup>			
Unfiltered water	4 (100%)	0/93	0.04

<sup>a</sup> Type of diarrhea (*n* = 102).

<sup>b</sup> Data on contact with animals and type of drinking water available in 97 patients only.



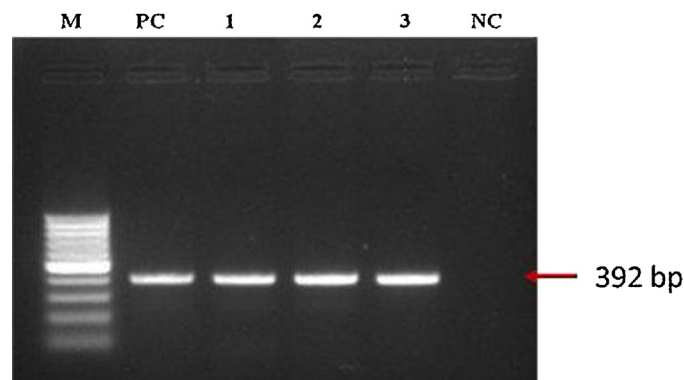


**Fig. 2** (a) An agarose gel showing PCR products of human fecal samples positive for microsporidia. Lane M, 50 bp ladder; Lane PC, positive control for microsporidia; Lanes 1–3, microsporidia-positive samples; Lane NC, negative control. (b) Digestion pattern of PCR products using the restriction enzymes HinfI and HindIII. Lane M, 100 bp ladder; Lane PC, positive control for *E. bieneusi* using HinfI; Lanes 2 and 4, samples positive for *E. bieneusi* using HindIII; Lane 3, samples positive for *E. bieneusi* using HinfI.

drinking water and contact with animals were available for 97 patients. All patients infected with microsporidia had unfiltered water (4/4, 100% vs. 0/93;  $p=0.04$ ). Other clinical manifestations including fever, cough, headache, chest pain, watery stool and contact with animals were comparable among patients with and without microsporidia. All four infected patients had a CD4 count  $<100$  cells/ $\mu$ l. Among them, two patients had CD4 cell counts  $\leq 100$  cells/ $\mu$ l, while two had CD4 counts  $\leq 50$  cells/ $\mu$ l.

### Species identification

A 1200 bp amplified fragment of microsporidia was observed using the specified primers (Fig. 2a). The amplified product was subjected to enzymatic digestion using the restriction enzymes HinfI and HindIII and displayed one restriction site with two bands of 230 bp, 940 bp and 386 bp, 784 bp, respectively (Fig. 2b). This pattern of bands suggests infection with *E. bieneusi*, which was confirmed by sequencing of PCR products in all patients.



**Fig. 3** An agarose gel showing secondary PCR amplified products of ITS of *E. bieneusi*. Lane M, 100 bp ladder; PC, positive control of ITS of *E. bieneusi*; Lanes 1–3, samples amplified for ITS of *E. bieneusi*; NC, negative control.

**Table 2** Polymorphic sites in the ITS of *E. bieneusi* genotypes L (consensus), Ind1, A (consensus), Ind2, Ind3 and Ind4.

Genotype	Nucleotide positions in ITS of <i>E. bieneusi</i>							
	5th	37th	73rd	135th	167th	228th	338th	339th
L consensus	A	T	G	A	A	T	C	T
Ind1	-	.	A	C	.	.	.	A
A consensus	A	T	G	T	A	T	A	T
Ind2	-	-	.	.	T	.	.	A
Ind3	-	.	.	.	.	-	.	A
Ind4	-	-	.	.	.	.	C	A

· Represents same base pair as that of consensus sequence; - represents a deletion as compared to the consensus sequences.

*E. intestinalis*, *E. hellem* and *E. cuniculi* were not detected in any patient.

### Genotyping of *E. bieneusi*

ITS sequences of *E. bieneusi* were amplified in the four patients (Fig. 3). *E. bieneusi* belonged to four distinct novel genotypes (Ind1 to Ind4). The four novel genotypes differed from each other by various single nucleotide polymorphisms in their ITS sequences. Genotype Ind1 showed 95% similarity with genotype L (AF267142.1) reported in cats from Germany with nucleotide changes at four positions (Table 2). Genotypes Ind2 to Ind4 showed 94–96% similarity to host-specific genotype A (AF101197.1) reported in humans with several nucleotide changes, as shown in Table 2.

### Nucleotide accession number assigned to genotypes of *E. bieneusi*

The gene bank accession numbers for sequences of genotypes of *E. bieneusi* submitted are KP325473–KP325476.

### Phylogenetic analysis

Fig. 4 shows the phylogenetic tree view, which depicts clustering of genotype Ind1 (1/4) with zoonotic genotypes of isolates reported in cats (AF267142.1), gorillas, wild boars, and cattle. Genotypes Ind2 to Ind4 (3/4) were grouped with host-specific genotypes of isolates reported in humans from Germany and Brazil.

### Discussion

In the present study, 1.8% of HIV-infected patients had microsporidia. All of the patients infected with

microsporidia had diarrhea and drank unfiltered water. All four patients infected with microsporidia had a CD4 cell count  $\leq 100$  cells/ $\mu$ l. All four patients were infected with *E. bieneusi*. Four distinct and novel genotypes, Ind1 to Ind4, were identified. Phylogenetic analysis of ITS sequences of *E. bieneusi* supports presumptive transmission mainly through the anthropological route among HIV-infected patients. However, the zoonotic route of transmission may also occur.

Microsporidia infect immunocompromised hosts, especially HIV-infected patients, worldwide. In the present study, 1.8% of HIV-infected patients had microsporidia. Similarly, a few other studies from India reported a prevalence ranging from 1% to 14% based on the microscopic and molecular diagnostic methods employed [2–5]. However, from other parts of the world, variable prevalences from as low as 0.1% to as high as 50% have been reported depending on variations in the study population and diagnostic methods employed [1]. The low prevalence reported in the current study could be due to empiric administration of anti-parasitic drugs to the patient in a developing nation such as India. In addition, administration of highly active anti-retroviral therapy (HAART) significantly reduces the risk of opportunistic infection among patients with HIV [16]. In the current study, the prevalence of microsporidia is comparatively low among HIV-infected patients. However, infection with microsporidia cannot be overlooked among HIV patients, as it leads to significant morbidity.

In the current study, all HIV patients infected with microsporidia had diarrhea. Similarly, in a study from India, HIV patients infected with microsporidia also presented with diarrhea [5]. In addition, other studies reported the association of diarrhea with microsporidia-infected HIV-positive patients from other parts of the world [7,17–20]. Thus, these studies support the association of microsporidia with diarrhea among HIV-infected



Fig. 4 Phylogenetic relationship of *E. bieneusi* inferred from the neighbor joining analysis of the ITS region of the rRNA gene.

patients. In our study, all four HIV patients infected with microsporidia had a CD4 count less than 100 cells/ $\mu$ l. This is in accordance with other studies reporting microsporidia among HIV-infected patients with low CD4 counts [20–24]. Therefore, HIV-infected patients having a CD4 cell count <100 cells/ $\mu$ l and presenting with diarrhea should be tested for infection of microsporidia.

In our study, infection with microsporidia was more prevalent in patients drinking unfiltered water. Similarly, in another study, microsporidia was detected in surface water [25]. In a study from France, a waterborne outbreak of intestinal microsporidiosis was reported in HIV-infected patients [26]. Therefore, we can speculate that contaminated water could be a source of microsporidia infection among HIV-infected patients. Therefore, drinking filtered water might decrease the occurrence of infection with microsporidia among HIV-infected patients.

In our study, *E. bienersi* was identified as the causative species in all patients infected with microsporidia. This is in accordance with previously published reports worldwide [2,20,27–30]. Conversely, in a study from India, *E. intestinalis* has been detected as the most common cause of infection among HIV-infected patients with diarrhea [5]. *E. bienersi* is the most common species infecting HIV patients [31]. The available drug of choice, albendazole is not effective against *E. bienersi*. Therefore, species differentiation is an important tool to administer appropriate treatment therapy to patients infected with microsporidia.

*E. bienersi* is the most prevalent species identified among HIV-infected patients. However, no genotypic studies on *E. bienersi* have been documented from a developing nation such as India. Genotyping would help establish transmission routes of microsporidia among humans. To the best of our knowledge, this is the first study on the molecular epidemiology of *E. bienersi* in HIV-infected patients from India. In the current study, four novel genotypes of *E. bienersi* (Ind1 to Ind4) were identified. Ind1 showed 95% similarity to genotype L. Genotype L with zoonotic potential is non-host specific and has been identified in *E. bienersi* isolates reported in cats from Germany [32]. In our study, genotype Ind1 was detected in a patient who was in contact with animals. There are several studies reporting the detection of microsporidia in feces of animals, including dogs, cats, cattle, and birds [33,34]. This suggests possible transmission of spores from animals to humans due to infected animals shedding spores of microsporidia in their feces. Ind2 to Ind4 showed 94–96% similarity to genotype A.

Genotype A is host specific and exclusively identified in humans. Genotype A was detected among HIV-infected patients from Peru and Thailand [15,35]. This further suggests human-to-human transmission of spores through enteric carriage. The phylogenetic tree constructed from ITS sequences of *E. bienersi* reported in our study aligned with earlier published sequences revealed the genetic relatedness between them. Genotype Ind1 from the present study was clustered together with genotype L reported in cats from Germany. Therefore, Genotype Ind1 from humans is non-host specific, infecting both humans and animals. Genotypes Ind2 to Ind4 were clustered together with genotype A, which are host specific, infecting humans only. Therefore, Ind2 to Ind4 could be stated as host-specific genotypes following the anthroponotic route of transmission. However, a large number of *E. bienersi*-positive samples should be studied to establish its molecular epidemiology in this geographical region, and further correlation with disease severity could be performed.

In conclusion, the prevalence of microsporidia among HIV infected patients is 1.8%. Microsporidia commonly causes diarrhea among HIV-infected patients with CD4 cell counts  $\leq$ 100 cells/ $\mu$ l. *E. bienersi* was the most common species identified among the studied HIV-infected patients. Four novel genotypes of *E. bienersi* were identified. Molecular epidemiology suggested presumptive transmission of *E. bienersi* mainly through the anthropological route among patients with HIV.

## Conflict of interest

All authors confirm that there is no conflict of interest.

## Ethical approval

The study protocol was approved by the Institutional Ethics Committee (PGI/DIR/RC/1085/2007).

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